page 12, lines 15-16; claims 102-106: page 13, lines 8-26; claims 107-109: page 14, lines 1-5; claims 110: page 43, lines 4-9 and page 53, line 35 to page 54, line 1; claim 111: see support for claim 97, page 15, lines 11-22; claim 112: see support for claim 97, page 73, lines 15-18; claim 113: see support for claim 97, page 15, line 26 to page 16, line 20; claims 114-116: page 17, lines 5-10; claims 117-118: page 18, lines 5-10. Claims 97-118 do not involve any issue of new matter such that entry of this amendment is respectfully requested.

Figure 6B

The Examiner stated the prior objection to the disclosure is maintained for the reasons as set forth in the last Office Action mailed 6/19/98 (see Paper No. 16). The Examiner stated applicants submit they will provide a new Figure 6B to overcome the rejection when the case is in condition for allowance. The Examiner stated until applicants submit a proper Figure said objection is maintained.

In response, applicants will submit a new figure 6B upon the indication of allowable subject matter.

Obviousness-type double patenting rejection

The Examiner provisionally rejected claims 69-96 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over the all the claims of copending Application No. 08/477,097.

The Examiner stated applicants assert that the added new claims in

the copending application obviate the obvious type double patenting since the claims in the 097' application are directed to a conjugation partner that is an immunogenic protein-based carrier. The Examiner stated applicants' arguments are not persuasive since BSA and KLH have long been used as immunogenic protein based carriers for the conjugation of substances to allow for the generation of specific immune responses. The Examiner stated these carriers have long been discussed in general textbooks such as Antibodies: A Laboratory Manual by Harlow and lane (1988, Cold Spring Harbor Laboratories, page 129). The Examiner stated the claims of the instant application encompass conjugating the ceramide portion GM2 via a variety of linkages as recited the claims in copending application 08/477,097 and the substitution of KLH for the a generic immunogenic protein-based carrier of the 097' application is an obvious variation. The Examiner stated that the application amendments are insufficient to remove the rejection.

The Examiner provisionally rejected claims 69-96 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims all the claims of copending Application Nos. 08/475,784. The Examiner stated although the conflicting claims are not identical, they are not patentably distinct from each other for the reason set forth in the prior Office Actions. The Examiner stated applicants argue that the 784' applications are directed to compositions which are gangliosides and not GM2 or GD2. The Examiner stated it is respectfully submitted that the ganglioside of the '784 application is selected from the group consisting of GM2... etc, as is admitted by applicants in the traversal of the double patenting rejection. The Examiner stated thus, the applications have clearly overlapping

subject matter in these two applications.

The Examiner provisionally rejected claims 69-96 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over all the claims of copending Application Nos. 08/481,809 in view of Livingston et al. (Cancer Research, 149:7045-7050, 1989. The Examiner stated although the conflicting claims are not identical, they are not patentably distinct from each other for the reason set forth below. The Examiner stated the '809 application claims compositions and methods using gangliosides in general linked via the ceramide portion of the ganglioside to KLH and to an adjuvant, a carbohydrate saponin and a phrmaceutically acceptable carrier. The Examiner stated the instantly claimed compositions drawn to specific species of gangliosides (GM2 and GD2) conjugated to KLH anticipate admixed with a carbohydrate saponin and a pharmaceutically acceptable carrier. The Examiner stated it would have been *prima facie* obvious to attach either of the gangliosides GM2 or GD2 through a ceramide carbon because Livingston et al teach that these are medically important gangliosides and immune responses generated against them are able to treat cancer and GM2 and GD2 are species of gangliosides.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove canceled claims 69-96 without prejudice to their right to pursue the subject matter of these claims in a later-filed application. Applicants contend that this amendment obviates the above rejection and respectfully

request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. 112, first paragraph

The Examiner rejected claims 69-71 and 72-96 under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the intention for the reasons set forth the Office Action mailed 6/13/96 (see Paper No. 8). The Examiner stated applicants arguments' have been carefully considered. Applicants' again argue make and test. The Examiner stated this is again not persuasive for reasons already extensively made of record in the previous response and reiterated below.

The Examiner stated protein chemistry is probably one of the most unpredictable areas of biotechnology. The Examiner stated for example, replacement of a single lysine residue at position 118 of the acidic fibroblast growth factor by glutamic acid to led to a substantial loss of heparin binding, receptor binding, and biological activity of the protein (see Burgess et al.). The Examiner stated in transforming growth factor alpha, replacement of aspartic acid at position 47 with alanine, or asparagine did not affect biological activity while replacement with serine or glutamic acid sharply reduce the biological activity of the mitogen (see Lazar et al.). Rudinger et al. Teachers "particular amino acids and sequences for different aspects of biological activity can not be predicted a *priori* but must be determined from case to case by painstakingly experimental study" (see page 6). The Examiner stated Salgaller et al teach modifications (i.e.

deletions) of the amino acid structure of peptide can alter the activity of the protein. The Examiner stated Fox et al. Teach methods for determining fragments which have antigenic activity is unpredictable. The Examiner stated these references demonstrate that a even a single amino acid substitution or what happens to be an inconsequential chemical modification, will often dramatically affect the biological activity of a protein. The Examiner stated in view of the lack of guidance, lack of examples, and lack of predictable associated with regard to producing and using the myriad or derivatives and fragments encompassed in the scope of the claims one skilled in the art would be forced into undue experimentation in order to practice broadly the claimed invention.

The Examiner stated that contrary to applicants' arguments it is reasonable to conclude an undue burden is required to screen for positions within the sequence where amino acid modification (i.e. additions, deletions, or modifications) can be made with a reasonable expectation of success in obtaining similar activity/utility are limited and the result of such modifications is unpredictable as exemplified by the teachings of Lazar et al. Burgess et al., Rudinger et al., and Salgaller et al.

The Examiner stated these references demonstrate that a even a single amino acid substitution or what appears to be an inconsequential chemical modification, will often dramatically affect the biological activity of a protein. The Examiner stated the specification does not support the broad scope of the claims which encompass a multitude of analogs or equivalents because the specification does not disclose the following: the general tolerance to modification and extent of such tolerance; specific

positions which can be predictably modified; and the specification provide essentially no guidance as to which of the essentially infinite possible choice is likely to be successful.

The Examiner stated thus, applicants have not sufficient guidance to enable one skilled in the art to make and use the claimed derivatives in a manner reasonably correlated with the scope of the claims broadly including any number of deletions, addition, and/or substitutions of any size. The Examiner stated the scope of the claims must bear a reasonable correlation with the scope of enablement (In re Fisher, 166 USPQ 19 24 (CCPA 1970). The Examiner stated without such guidance, the changes wich can be made and still maintain activity/utility is unpredictable and the experimentation left to those skilled in the art is unnecessarily, and improperly, extensive and undue. See Ex parte Forman , 230 U.S.P.Q. 546 (Bd. Pat. App. & Int. 1986).

The Examiner stated that applicants cite to page 12, lines 4-13 of the specification for support of using derivatives of KLH. The Examiner stated that said disclosure is not commensurate in scope with the claimed invention. The Examiner stated that said cite makes reference only to linking KLH to an "immunological adjuvant" an not amino acid modifications (i.e deletions, substitutions) of KLH. The Examiner stated as set forth above the scope of the claims must bear a reasonable correlation with the scope of enablement (In re Fisher, 166 USPQ 19 24 (CCPA 190)). The Examiner stated for the reason set forth above and in the last Office Action said rejection is maintained.

In response, applicants respectfully traverse the Examiner's above

rejection. Applicants contend that the claimed invention was enabled. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove canceled claims 69-96. Applicants contend that the invention of newly submitted claims 97-118 is enabled. Applicants contend that this amendment obviates the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

claims 81-86

The Examiner stated as to claims 81-86, the claims are enabled for the use of the composition only for treatment of cancer but are NOT enabled for the prevention of cancer, for reasons made of record in Paper No. 8, mailed 6-13-96.

In response, applicants respectfully traverse the Examiner's above rejection. Applicants contend that a claim directed to the prevention and treatment of a cancer using the subject composition is fully enabled. In support of claims concerning the treatment and/or prevention of the cancer, applicants attach hereto as **Exhibit A** a copy of the following paper: Helen Zhang et al "Antibodies against GD2 Ganglioside Can Eradicate Syngeneic Cancer," Cancer Research 58: 2844-2849 (1998). This paper demonstrates that the conjugated vaccine of the subject invention prevents the outgrowth of micrometastases (see page 2844, first column). The paper shows that the conjugated vaccine prevents establishment of subsequently administered EL4 challenge (which is a lymphoma), and eliminates EL4 micrometastases when administered after EL4 challenge (see page 2844, second column). The paper shows that mice receiving the conjugate vaccine survived significantly longer, and that one mouse did not show any evidence of tumor (see page 2845, second column). The paper teaches that the conjugated vaccine

protects against tumor challenge and eliminates micrometastases. Applicants contend that this is support for the use of the composition to treat or prevent cancer. Applicants contend that these remarks obviate the above rejections and respectfully request that the Examiner reconsider and withdraw the rejection.

Rejection under 35 U.S.C. 112, first paragraph

The Examiner rejected claims 69-96 under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner stated this is a new matter rejection. The Examiner stated applicants point to page 32, lines 13-18 and page 12, lines 22-26 for support for the now claimed invention. The Examiner stated this is not persuasive, the passage at page 32, lines 13-18 provide for a *specific coupling procedure at the C-4 carbon of the sphingosine moiety of the ceramide to the ε-aminolysyl group of a proteins*(ozonolysis, production of a functional aldehyde group and coupling to an ε-aminolysyl group on a protein by reductive amination. The Examiner stated the passage at page 12, lines 22-26 in combination with the passage at page 32, lines 13-18 does not support a broad coupling to any generic portion of the ceramide backbone of the ganglioside, by any generic means by cleavage of any double bond (i.e. C=O) and coupling by any linkage process. The Examiner stated the written description, convey that applicants had at the time filing contemplated any means of coupling to any portion of the ceramide, a concept that is now broadly claimed. The Examiner stated applicants were clearly not in possession of that which is now broadly claimed. The Examiner stated correction

is required.

In response, applicants respectfully traverse the Examiner's above rejection. Applicants contend that they were in possession of the claimed invention at the time of filing. Nevertheless, without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove canceled claims 69-96 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application. Applicants have also hereinabove added new claims 97-118 which recite that "the ganglioside derivative is conjugated to Keyhole Limpet Hemocyanin or the derivative thereof through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin or the derivative thereof." Applicants respectfully direct the Examiner's attention to page 32, lines 15-20 for support for this claim language. Applicants contend that this amendment obviates the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. 103(a)

The Examiner rejected claims 69-81 and 83-96 under 35 U.S.C. 103(a) as being unpatentable over Livingston et al. (Cancer Research, 149:7045-7050, 1989) in view of Ritter et al. (Seminars in Cancer Biology, 2:401-409, 1991), Liane et al (Journal of Biological Chemistry, 249(14):4460-4466, 1974), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (Immunobiol, 182:32-43, 1990), Kensil et al. (The Journal of Immunology, 146(2):431-437, 1991), and Marciani et al. (Vaccine, 9:89-96, 1991) and Uemura et al (J. Biochem, 79(6):1253-1261, 1976). The Examiner stated that Livingston et al (Cancer Research) teach a composition administered

to melanoma patients for stimulation the production of antibodies directed against a carbohydrate epitope on the ganglioside, GM2 (page 7046-7048). The Examiner stated Livingston et al teach that the composition for treatment is administered at a concentrations of 100, 200, or 300 ug with an adjuvant, Bacillus-Calmette-Geurin (BCG), and a pharmaceutically acceptable vehicle, phosphate buffered saline, (p 7046, column 1, paragraph 1 and column 2, paragraph bridging p 7046-47). The Examiner stated Livingston et al teach that melanoma recurrence was delayed in patients developing GM2 antibodies after treatment with the composition (page 7048, paragraph 1 and column 2, paragraph 2). The Examiner stated Livingston et al teach that more patients produced IgM antibodies than IgG antibodies to the GM2 (pate 7047, paragraph bridging columns 1-2). The Examiner stated Livingston et al also teach the gangliosides GM2, GD2 and GD3 are expressed on the cell surface of human malignant melanomas (page 7045, column 1, paragraph 2). The Examiner stated Livingston et al differ by not teaching the conjugation of the GM2 or other gangliosides by means of a carbon on the ceramide moiety with aminolysyl groups on Keyhole Limpet Hemocyanin (KLH) in composition and using this composition for treatment.

The Examiner stated Ritter et al (1991) teach that IgG responses to gangliosides may be increased by the covalent attachment of foreign carrier proteins such as KLH to the gangliosides resulting in the T cell help necessary for the response (page 406, paragraph 1). The Examiner stated Ritter et al teaches discloses that the advantage of inducing an IgG antibody response (vs IgM) against gangliosides is that IgG: a) has a higher affinity, b) is better able to penetrate solid tissues, c) is able to mediate antibody-

dependent cell-mediated cytotoxicity, d) and is generally detectable in the serum for longer periods after immunization.

The Examiner stated that Liane et al (Journal of Biological Chemistry, 249(14):4460-4466, 1974) teach a method for covalent coupling of gangliosides to aminoethyl agarose or the amino group bearing glass beads by oxidative ozonolysis of the olefinic bond of the sphingosine moiety (i.e. the instant carbon double bond of ceramide) and coupling of the carboxyl bearing product to the amino group of aminoethyl agarose or the amino group bearing glass beads.

The Examiner stated that Ritter et al (1990) teach that GD3 lactone is more immunogenic than GD3.

The Examiner stated Livingston et al (U.S. Patent No. 5,102,663) teach that gangliosides GM3, GM2, GD3, GD2, GT3 and O-acetyl GD3 are gangliosides that are prominent cell-membrane components of melanoma and other tumors of neuroectodermal origin (column 1, lines 22-28).

The Examiner stated Liane et al (Journal of Biological Chemistry, 249(14):4460-4466, 1974) teach a method for covalent coupling of gangliosides to aminoethyl agarose or the amino group bearing glass beads by oxidative ozonolysis of the olefinic bond of the sphingosine moiety (i.e. the instant carbon double bond of ceramide) and coupling of the carboxyl bearing product to the amino group of aminoethyl agarose or the amino group bearing glass beads.

The Examiner stated Kensil et al teach that QS-21 (i.e. the instant carbohydrate derivable from the bark of a Quillaja saponaria Molina

tree) produced a higher antibody response than conventional aluminum hydroxide (page 433, column 2, paragraph 4, and Figure 3). The Examiner stated Kensil et al also teach that the immune responses obtained with QS-21, reached a plateau at doses between 10-80 ug in mice (page 433, column 1, paragraph 3).

The Examiner stated Maricani et al teach that the use of QS-21 adjuvant was useful because it did not cause a toxic reaction in cats (page 93, paragraph 1).

The Examiner stated Uemura et al (J Biochem, 79(6):1253-1261, 1976) teach that the ozonolysis and reduction of various shingolipids did not affect the haptenic reactivity of the ganglioside derivative with antibodies.

The Examiner stated that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to modify the composition taught by Livingston et al by conjugating the GM-2 to KLH by covalently coupling GM2 to KLH by substituting GM2 for the globoside and KLH for the aminoethyl agarose to produce a GM-2-KLH conjugate for use as a vaccine because the conjugated composition would be expected to enhance the IgG response to the ganglioside, as taught by Ritter et al (1991), thus providing the advantages by Ritter et al (1991) and adding the QS-21 would be advantageous because it provides for a higher antibody response that the commonly used adjuvant use by Kensil et al and QS-21 provides the advantages that it is not toxic to animals as is taught by Marciani et al. The Examiner stated it also would have been *prima facie* obvious to use doses of between 10 and 80 ug of QS-21 in the composition and optimize the dose accordingly because

the immune response with QS-21 plateaus at doses between 10-80 ug and optimization of the weight ratio of the components of the composition to provide an optimal response is well within the ordinary skill in the art and use the composition as modified supra for treatment of melanoma as taught by Livingston et al (Cancer Research). The Examiner stated it also would have been *prima facie* obvious to one of ordinary skill in the art to substitute any one of GM3, GD2, GD3, or O-acetyl GD3 for the GM2 ganglioside in the composition and method as combined supra because they are all prominent cell-membrane components of melanomas as taught by Livingston et al (U.S. Patent No. 5,102,663) and one of ordinary skill in the art would react with the melanomas cells. The Examiner stated it would have also been *prima facie* obvious to one of ordinary skill in the art would react with the melanoma cells. The Examiner stated it would have also been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the GD3, as taught by Ritter et al (1990) and would be expected to product an enhanced antibody response as compared to GD3. Optimization of the dosage, route of immunization, number of sites of immunization to administer the composition is will within the skill of the ordinary artisan. The Examiner stated one would have reasonably expected the conjugation procedure to work as substituted because conjugation through the ε-aminolysyl groups of carrier proteins for enhance immunogenicity is routine in the art and Uemura et al (J Biochem, 79(6):1253-1261, 1976) teach that the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity with antibodies.

The Examiner rejected claim 82 under 35 U.S.C. 103(a) as being unpatentable over Livingston et al. (Cancer Research), Ritter et

al. (Cancer Biology, 1991), Liane et al (Journal of Biological Chemistry, 249(14):4460-4466, 1974), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (1990), Kensil et al, and Marciani et al., and Uemura et al (J Biochem, 79(6):1253-1261, 1976) as applied to claims 69-81 and 83-96 above and further in view of Irie et al. (U.S. Patent No. 4,557,931).

The Examiner stated the teachings of Livingston et al. (Cancer Research), Ritter et al. (Cancer Biology, 1991), Liane et al (Journal of Biological Chemistry, 249(14):4460-4466, 1974), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (1990), Kensil et al, and Marciani et al., and Uemura et al (J Biochem, 79(6):1253-1261, 1976) are set forth supra. The Examiner stated the combination differs by not teaching the administration of the composition for treating cancer of epithelial origin or for producing antibodies to gangliosides found in the stroma of cancer. The Examiner stated that Irie et al teaches that the ganglioside GM2 is found on or in tumors of a variety of histological types including melanoma and breast carcinomas (column 1, lines 28-31). The Examiner stated that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to administer the GM-2-KLH conjugate/QS-21 composition or other ganglioside conjugate/QS-21 composition as combined *supra* to patients afflicted with or susceptible to a recurrence of cancer of an epithelial origin (i.e. breast carcinomas) because the ganglioside GM-2 is found in the stroma of the tumor as taught by Irie et al and one of ordinary skill in the art would expect that the antibodies produced by the composition react with the tumor and treat the disease.

In response, applicants respectfully traverse the Examiner's above rejection. Applicants contend that the cited references do not render obvious the claimed invention. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove canceled claims 69-96 without prejudice to their right to pursue the subject matter of these claims in a later-filed application. Applicants have hereinabove added new claims 97-118 which recite that the composition comprises: a) a conjugate of i) a GM2 or GD2 ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphingosine base, to ii) Keyhole Limpet Hemocyanin or a derivative thereof comprising an ε-aminolysyl group; b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and c) a pharmaceutically acceptable carrier; the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in a subject, wherein in the conjugate the ganglioside derivative is conjugated to Keyhole Limpet Hemocyanin or the derivative thereof through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin or the derivative thereof. Applicants contend that the cited the cited references, alone or in combination, do not teach or suggest applicant's claimed invention. Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Summary

For the reasons set forth hereinabove, applicants respectfully request that the Examiner reconsider and withdraw the various grounds for objection and rejection and earnestly solicit allowance of the now pending claims.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone at the number provided below.

No fee, other than the enclosed $565.00 fee which includes the $435.00 fee for a three-month extension of time and the $130.00 fee for additional claims, is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

John P. White
Registration No. 28,678
Attorneys for Applicant(s)
Cooper & Dunham, LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

4/6/00
John P. White Date
Reg. No. 28,678

[CANCER RESEARCH 58, 2844–2849, July 1, 1998]

Antibodies against GD2 Ganglioside Can Eradicate Syngeneic Cancer Micrometastases[1]

Helen Zhang, Shengle Zhang, Nai-Kong V. Cheung, Govindaswami Ragupathi, and Philip O. Livingston[2]

Departments of Medicine [H. Z., S. Z., G. R., P. O. L.] and Pediatrics [N-K. V. C.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

After 10 years of clinical trials in patients with advanced cancer, monoclonal antibodies (mAbs) against cell surface antigens have not lived up to their initial promise. One such cell surface antigen is the ganglioside GD2. GD2 is richly expressed at the cell surfaces of human neuroblastomas, sarcomas, and melanomas. We have described a murine lymphoma (EL4) that is syngeneic in C57BL/6 mice and expresses GD2, a mAb against GD2 (mAb 3F8), and we have prepared a conjugate vaccine (GD2-keyhole limpet hemocyanin plus immunological adjuvant QS-21) that consistently induces antibodies against GD2. We demonstrate here, for the first time in a syngeneic murine model, that passively administered and vaccine-induced antiganglioside antibodies prevent outgrowth of micrometastases, and we use this model to establish some of the parameters of this protection. The level of protection was proportional to antibody titer. Treatment regimens resulting in the highest titer antibodies induced the most protection, and protection was demonstrated even when immunization was initiated after tumor challenge. Treatment with 3F8 1, 2, or 4 days after i.v. tumor challenge was highly protective, but waiting until 7 or 10 days after challenge resulted in minimal protection. The results were similar whether number of liver metastases or survival was used as the end point. These results suggest that unmodified mAbs or antibody-inducing vaccines against GD2 (and possibly other cancer cell surface antigens) should be used exclusively in the adjuvant setting, where circulating tumor cells and micrometastases are the primary targets.

INTRODUCTION

Most mAb[3] treatments have been performed on patients with advanced disease, and the treatments were of short duration, with response of measurable disease as the end point. Responses have been rare. Occasional regression of measurable neuroblastoma, melanoma, and breast cancer lesions and more frequent regression of B-cell lymphomas have resulted in patients treated with mAbs against cell surface antigens, including: gangliosides GM2[4] (1), GD2 (2–5), and GD3 (6–8); HER2 neu (9); and lymphoma idiotype antigens (10, 11). Trials with mAbs against GD2 are a case in point. The response rate in children with GD2-positive cancers (primarily neuroblastomas) treated with mAb 14.G2a or 3F8 is between 0 and 25% (12, 13), and in melanoma patients treated with mAb 3F8, 14.G2a, or chimeric 14.18, the response rate is between 0 and 22% (13, 14). A chimeric 14.18-interleukin 2 fusion protein shown to be potent in a *scid/scid* xenograft model (15) is now being considered for clinical trials. Neither immunogenic GD2 vaccines nor a syngeneic animal model has been previously available, making it difficult to compare these various approaches or to test the many variables associated with antibody-mediated therapies in the setting of a normal immune system.

As opposed to the minimal benefit seen with mAbs in patients with advanced disease, there is an expanding body of evidence indicating that antibodies can protect against subsequent tumor challenge in experimental animals and prevent tumor recurrence in humans. mAbs against several protein or glycoprotein tumor antigens have resulted in significant protection from syngeneic tumors in the mouse (16–19), mAb R24 against GD3 has resulted in protection from syngeneic melanoma growth in hamsters (20), and mAbs against GD2/GD3 (21) or GD2 (22) have resulted in protection against human tumor challenges in nude mice. There is also evidence in humans that natural antibodies, passively administered antibodies, or vaccine-induced antibodies against cancer antigens can result in prolonged disease-free and overall survival in the adjuvant setting. (*a*) Paraneoplastic syndromes have been associated with high titers of natural (not induced by vaccine or passive administration) antibodies against onconeural antigens expressed on neurones and certain malignant cells. The antibodies are apparently induced by tumor growth and have been associated with autoimmune neurological disorders and, in addition, with delayed tumor progression and prolonged survival (23–25). (*b*) Patients with American Joint Commission On Cancer stage III melanoma and natural antibodies against GM2 ganglioside studied at two different medical centers have an 80–90% 5-year survival, compared to the expected 40% rate (26, 27). (*c*) Patients with small cell lung cancer and natural antibodies against small cell lung cancer had prolonged survival, compared to antibody-negative patients (28). (*d*) Patients with Dukes' C colon cancer treated with mAb 17-1A in the adjuvant setting had a significantly prolonged disease-free and overall survival, compared to randomized controls (29). (*e*) Antibody responses induced by vaccines in the adjuvant setting have been correlated with subsequent prolonged disease-free and overall survival (26, 27, 30–33).

Given the potential clinical importance of a variety of cell surface antigens, including ganglioside GD2, as targets for mAbs and cancer vaccines inducing an antibody response, we have identified a suitable syngeneic mouse model to address some of the variables associated with antibody-mediated protection from and therapy of cancer. EL4 is a lymphoma syngeneic in C57BL/6 mice that we have previously reported to express GD2 (34). It is a unique model, in that GD2 is also a human tumor antigen, against which there is not only a clinically active mAb but also a consistently immunogenic conjugate vaccine, GD2-KLH plus QS21. We demonstrate here that passively administered and vaccine-induced antibodies are able to prevent establishment of subsequently administered EL4 challenge and to eliminate EL4 micrometastases when administered after EL4 challenge, and we define some of the parameters of this protection.

MATERIALS AND METHODS

mAbs and Vaccine

The origins of mAb 3F8 (IgG3) against GD2 (35), mAb 696 (IgM) against GM2 (36), mAb 013 against a primitive human neuroectodermal bone tumor (37), and mAb 1E3 against Tn antigen (38) have been described. Neither 013

Received 1/12/98; accepted 4/28/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by NIH Grants PO1 CA 33049 (to P. O. L. and G. R.) and RO1 CA 61017 (N-K. V. C.). P. O. L. is a paid consultant to and has share options in Progenics Pharmaceuticals, Inc., who has licensed the GD2-KLH vaccine from Memorial Sloan-Kettering Cancer Center. In exchange for the rights conveyed under the licensing agreement, Memorial Sloan-Kettering Cancer Center received shares in Progenics Pharmaceuticals, Inc., in lieu of a licensing fee.

[2] To whom requests for reprints should be addressed, at Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Fax: (212) 794-4352.

[3] The abbreviations used are: mAb, monoclonal antibody; KLH, keyhole limpet hemocyanin; CDC, complement-dependent cytotoxicity.

[4] The designations GM2, GD2, and GD3 are used in accordance with the abbreviated ganglioside nomenclature of Svennerholm (48).

Table 1 *Experiment 1: liver metastases after i.v. challenge with EL4 lymphoma incubated with mAbs 3F8 (against GD2) and 696 (against GM2)*[a]

mAb	No. of mice	No. of tumors in liver	Liver mass (g)
PBS (control)	8	57.8 ± 67.2	2.59 ± 1.03
mAb 696	5	94 ± 100	2.52 ± 1.21
mAb 3F8	5	0	1.23 ± 0.04[b]
mAb 696 + mAb 3F8	5	0	1.20 ± 0.14[b]

[a] After incubation with 100 μg/ml 3F8 and 50 μg/ml 696 for 1 h, 3×10^4 EL-4 cells per mouse were injected (i.v.) into C57BL/6 mice. Thirty-four days after challenge, mice were sacrificed, and the livers were evaluated. Results are expressed as mean ± SD.
[b] $P < 0.01$, compared with PBS control group.

nor IE3 reacts with EL4. Immunological Adjuvant QS-21, a purified saponin fraction (39), was obtained from Aquilla Biopharmaceuticals Inc. (Worcester, MA). GD2 and GD2 conjugated to KLH were provided by Progenics Pharmaceuticals Inc. (Tarrytown, NY). Conjugation of GD2 to KLH was achieved by conversion of the GD2 ceramide double bond to aldehyde by ozonolysis and attachment to KLH by reductive amination in the presence of cyanoborohydride, as described previously for GD3 (40). Each GD2-KLH vaccine contained 10 μg of GD2 conjugated to 60 μg of KLH, plus 10 μg QS-21. Vaccines were administered s.c. three times at 1-week intervals, except in the final experiment, when they were administered at 4-day intervals.

Mice and Cell Lines

C57BL/6 mice (6 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The *EL4* cell line was established from lymphoma induced in a C57BL/6 mouse by 9,10-dimethyl-1,2-benzanthracene. It has recently been shown to express GD2 ganglioside (34). *EL4* was maintained in 10% FCS-RPMI. For tumor cell challenges, *EL4* cells were washed three times in PBS, and 3×10^4 cells (in the final experiment, 5×10^3 cells) were injected i.v. into the tail vein. At the indicated time points, mice were sacrificed, and livers were removed, weighed, and fixed in 10% formalin. Metastases were also frequently present in lymph nodes and other sites (although rarely in the lungs), but hepatic metastases were easiest to quantitate. Hepatic metastases were detected as white nodules on the liver surface.

Serological Assays

ELISA. ELISAs were performed as described previously (41). GD2 or GM2 in ethanol was coated on ELISA plates at 0.1 μg/well. A series of antiserum dilutions were incubated with the coated ganglioside for 1 h. Secondary antibodies were alkaline phosphatase-conjugated goat antimouse IgG or IgM at a dilution of 1:200 (Southern Biotechnology Associates, Inc., Birmingham, AL). ELISA titer is defined as the highest dilution yielding an absorbance of 0.1 or greater over that of normal control mouse sera. mAbs 3F8 and 696 were used as positive controls in each assay.

Flow Cytometry. *EL4* cells (3×10^5) were incubated with 40 μl of 1:30 diluted antisera or 1:2 diluted mAb supernatant for 30 min on ice. After washing with 3% FCS in PBS, the cells were incubated with 20 μl of 1:15 diluted FITC-labeled goat antimouse IgG or IgM (Southern Biotechnology Associates, Inc.). The positive population of the stained cells was quantitated by flow cytometry (EPICS-Profile II; Coulter Co., Hialeah, FL), as described previously (41).

CDC. In 100 μl of 5% FCS in RPMI, 2×10^5 *EL4* cells were incubated with 10 μl of 1:10 mouse antiserum or 10 μg/ml mAb for 10 min. Thirty μl of complement (guinea pig; Sigma Chemical Co.) were added and incubated at 37°C for 4 h. Thirty μl of 0.4% trypan blue were added, and after 3 min, dead and viable cells were counted (41).

Statistical Methods

Experimental groups were compared to controls for number of hepatic metastases, survival, or antibody titers using the Mann-Whitney two-sample *t* test (42).

RESULTS

Having previously shown that mAb 3F8 was able to bind to EL4 and induce potent CDC and antibody-dependent cell-mediated cytotoxicity (3, 13, 35), we performed a series of experiments progressively testing the ability of passively administered and then actively induced antibodies against GD2 to eradicate hepatic micrometastases (experiments 1 and 2) and to prolong survival (experiments 3–7).

Effect of mAb Administration on Hepatic Metastases (Experiments 1 and 2). In experiment 1, we mixed 3F8 or negative control mAb 696 with the *EL4* lymphoma cells prior to challenge to confirm *in vivo* impact of antibody binding. EL4 cells were incubated for 1 h with PBS, mAb 696 (against GM2, which is minimally expressed on *EL4*), mAb 3F8, or mAbs 696 and 3F8 prior to i.v. challenge. All mice were sacrificed on day 34, hepatic metastases were counted, and livers were weighed (Table 1). Only *EL4* preincubation with 3F8 ± 696 eliminated metastases. In experiment 2, mice were injected i.v. with PBS, negative control antibody IE3 (100 μg), or one of three doses of 3F8 (50, 100, or 250 μg) 2 h before i.v. challenge with untreated *EL4* cells. Mice were sacrificed at day 30. Administration of all three doses of 3F8 eliminated metastases in most mice (Table 2).

Effect of mAb Administration or Vaccination on Survival (Experiments 3–6). In experiment 3, two groups of six mice received a single i.v. injection of 200 μg of 3F8 1 day before or 2 days after EL4 i.v. challenge. Three additional groups of six mice were vaccinated three times (on days −21, −14, and −7) prior to *EL4* challenge. They were vaccinated with PBS, 10 μg of GD2 mixed with 60 μg of KLH plus QS21 (negative controls), or 10 μg of GD2 conjugated to 60 μg of KLH plus QS21. Mice receiving the conjugate vaccine survived significantly longer than did the control mice ($P < 0.008$), and one mouse was sacrificed on day 100 with no evidence of tumor. Five of six mice receiving 3F8 1 day before challenge and five of six mice receiving 3F8 2 days after challenge also remained tumor free (Fig. 1, *Experiment 3*). All negative control mice died by day 28.

Experiments 4 and 5 focused on treatment with mAb. In experiment 4, groups of four or five mice received PBS or 3F8 2 days or 4 days after EL4 challenge i.v. All 3F8-treated mice survived longer than did control mice ($P < 0.004$), and three mice in the 3F8 groups remained tumor free (Fig. 1, *Experiment 4*). Experiment 5 compared treatment with PBS or mAb O13 (negative controls) and treatment with 50 or 200 μg of 3F8, all administered 2 days after *EL4* challenge i.v. (Fig. 1, *Experiment 5*). Once again, all 3F8-treated mice survived longer than did any control mouse ($P < 0.004$), and most mice (8 of 12) treated with either dose of 3F8 remained tumor free.

Experiment 6 again compared immunization prior to tumor challenge with mAb treatment at various intervals after challenge. All vaccinated mice again survived longer than did any control mouse ($P < 0.004$), and four of six mice remained disease free (Fig. 1, *Experiment 6b*). Most mice receiving 70 μg of 3F8 2 or 4 days after challenge remained disease free. However, the same dose 7 or 10 days after challenge had no significant effect (Fig. 1, *Experiment 6a*). Experiment 6 was a single experiment but is presented in two panels for greater clarity. Once again, the relevant negative control treatments (mAb R24 against GD3, which is not expressed on *EL4*, and

Table 2 *Experiment 2: liver metastases after i.v. injection of mAbs followed by i.v. EL4 challenge*[a]

Treatment	No. of mice	No. tumors in liver	Liver mass (g)
PBS (control)	7	29.2 ± 14.8	1.90 ± 0.47
mAb IE3 (100 μg/mouse)	9	17.6 ± 15.9	1.95 ± 0.72
mAb 3F8 (50 μg/mouse)	6	0[b]	1.03 ± 0.13
mAb 3F8 (100 μg/mouse)	6	4.3 ± 7.0[c]	1.17 ± 0.41
mAb 3F8 (250 μg/mouse)	6	0[d]	0.90 ± 0.16

[a] Challenge was with 3×10^4 EL-4 cells 2 h after mAb injection. The mice were sacrificed 30 days after challenge, and the livers were evaluated. Results are expressed as mean ± SE.
[b] $P < 0.01$, compared with PBS control group.
[c] $P < 0.02$, compared with PBS control group.
[d] $P < 0.001$, compared with PBS control group.

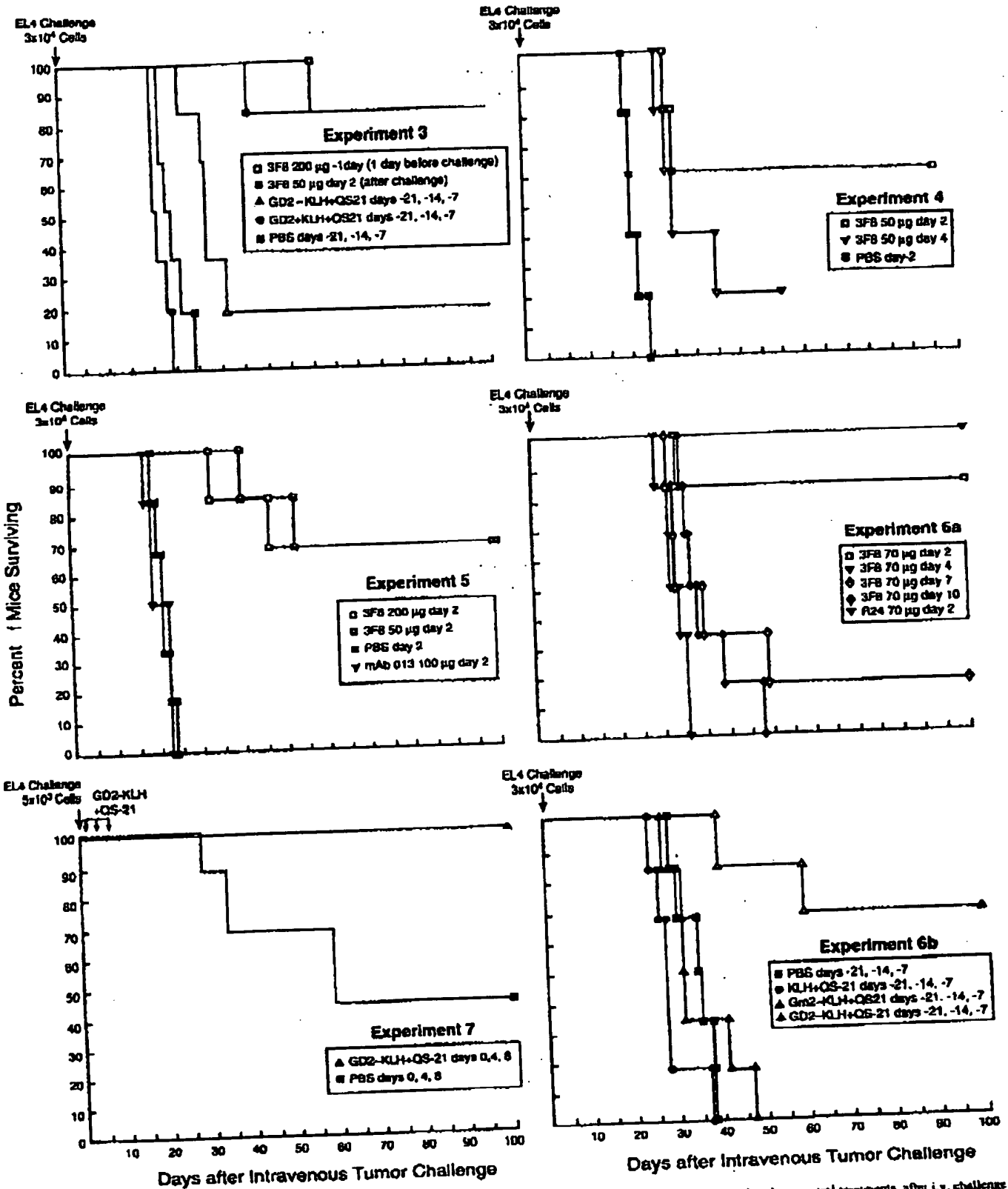

Fig. 1. Survival of groups of four to six mice treated in five separate experiments with 3F8 mAb, GD2-KLH plus QS-21 vaccine, and various control treatments, after i.v. challenge with syngeneic EL4 lymphoma cells. 3F8 mAb against GD2 administered prior to challenge or 1–4 days after challenge and GM2-KLH plus QS-21 vaccination prior to challenge or starting immediately after challenge were both protective.

Table 3 *Antibody reactivity in sera of mice treated with GD2-KLH vaccine or mAb 3F8*[a]

Treatment	No. of mice	Reciprocal ELISA titer		Flow cytometry (% positive cells)		CDC (% dead cells)
		IgM	IgG	IgM	IgG	
Experiment 3						
PBS (100 μl)	6	0/0	0/0	3.1–3.3/3.2	3.1–3.4/3.2	10–10/10
GD2 + KLH + QS21 (10 μg + 60 μg + 10 μg)	6	0–80/40	0	5.0–8.9/6.5	4.2–5.5/4.5	Not tested
GD2-KLH + QS21	6	40–640/80	80–2,560/640	18–88/57	22–93.6/60.5	20–60/40
3F8 (250 μg), 1 day before challenge	6	0/0	2,560–5,120/5,120	Not tested	99–99.3/99	90–95/95
Experiment 6						
PBS (100 μl)	6	0/0	0/0	1.5–2.9/2.0	1.4–2.6/1.7	10–15/10
KLH + QS21 (60 μg + 10 μg)	6	0/0	0/0	1.3–8.1/3.6	1.3–2.2/2.0	10–15/10
GD2-KLH + QS21	6	160–640/320	180–14,580/1,620	57–99/95	13–99/89	40–80/60
3F8 (250 μg)	6	0/0	1,620–4,860/3,240	Not tested	98–100/99	85–95/95

[a] Mice were bled 7 days after the third immunization with GD2 vaccine or 4–5 days after mAb 3F8 injection. Results are expressed as range/median.

vaccination with KLH plus QS21, GM2-KLH plus QS21, and PBS, which do not induce anti-GD2 antibodies) had no effect.

Correlation between Serum Antibody Titer and Survival. Serum anti-GD2 antibody titers immediately after 3F8 administration were not tested, but they ranged between 1:1620 and 1:4860 (median, 1:4860) 3–5 days later, except in experiment 4, in which they were between 1:180 and 1:4860 (median, 1:540). Vaccine-induced antibody titers ranged between 1:640 and 1:1620 for IgG and 1:80 and 1:1620 for IgM (Table 3). Comparable antibody titers by ELISA resulted in comparable reactivity by flow cytometry and complement-mediated cytotoxicity, whether due to 3F8 or vaccine administration. In both cases, protection from subsequent tumor challenge resulted. A correlation between antibody titer and *in vivo* protection is suggested by these results. Administration of 3F8 resulted in higher serum titers against GD2 than vaccine administration in both experiments ($P < 0.004$ for CDC) and greater protection ($P < 0.008$ for experiment 3). In experiment 4, in which 3F8 levels were lower than expected after 3F8 administration, survival was lower as well. Vaccine-induced antibody titers prior to challenge were higher in experiment 6 than in experiment 3, and protection was greater as well ($P < 0.025$).

Therapeutic Vaccination. Because 3F8 administration 7 or 10 days after EL4 challenge with 3×10^4 resulted in minimal protection, this suggested that vaccination after challenge, which was normally performed at weekly intervals and required 14–21 days for antibody induction, would be ineffectual. Consequently, we performed one final experiment aimed at testing the ability of vaccinations started after tumor challenge to prolong survival. In experiment 7, the number of EL4 cells per challenge was decreased from 3×10^4 to 5×10^3 cells, and the vaccines were administered on days 0, 3, and 7, beginning immediately after the challenge. Median IgM and IgG antibody titers on days 13 and 18 were both 1:320. Protection was again seen (Fig. 1, *Experiment 7*), although the difference was not statistically significant ($P = 0.15$).

DISCUSSION

The mechanism of antibody effect against bacteria is predominantly complement mediated inflammation and cytotoxicity (CDC: Ref. 43). Although other effector mechanisms have been suggested for GD2 antibody, such as inhibition of tumor cell substratum or extracellular matrix interactions (22), activation of immune effector mechanisms remains the most likely explanation. 3F8, the anti-GD2 mAb used here, is an IgG3 antibody that is particularly potent at inducing complement-mediated inflammation/cytotoxicity and antibody-dependent cell-mediated cytotoxicity. We have previously demonstrated, in melanoma patients, that natural or vaccine-induced IgM antibodies against GM2 ganglioside correlated with improved disease-free and overall survival (26, 44) and that a GM2-KLH plus QS21 vaccine induced IgM and IgG antibodies in melanoma patients, which were both able to mediate CDC (45). Fortuitously, the IgG subclasses were IgG1 and IgG3 (44–46), the two human subclasses best able to mediate CDC. The same applies to the murine model we describe here. IgM and IgG antibodies were induced in all vaccinated mice, these antibodies and administered 3F8 mAbs were able to mediate potent CDC, and antibody titers correlated with survival and inversely with the number of hepatic metastases. Although mAbs administered up until 4 days after challenge were able to completely prevent tumor growth in most mice, by 7–10 days after challenge, 3F8 administration had little effect. This strongly suggests that treatment with mAbs or vaccines inducing antibodies must be restricted to the adjuvant setting, where the targets are circulating tumor cells and micrometastases, and it may explain why mAb treatment trials in patients with measurable tumor burdens have not been more successful.

Passively administered and vaccine-induced antibodies were both able to protect against growth of micrometastases. There are advantages and disadvantages to each approach. Therapy in the adjuvant setting may require repeated treatments to maintain antibody titers over a prolonged period to overcome the issue of tumor cell dormancy and sanctuary sites. Except in immunosuppressed patients, this excludes murine mAbs, which would be eliminated within weeks by human antimouse antibodies. Chimeric, humanized, or human mAbs would overcome this issue but would be subject to elimination by anti-idiotypic antibodies. On the other hand, in the absence of human antimouse antibodies or anti-idiotypic antibodies, higher serum antibody levels than could be induced by vaccination are assured after mAb administration, and such antibodies have been or could be produced against most antigens. Vaccines against most defined tumor antigens are more practical to produce and administer because they can be administered s.c. and at longer intervals. Phase III trials with GM2-KLH and sialyl Tn-KLH vaccines that consistently induce moderate titers of antibodies against these antigens are currently ongoing in the adjuvant setting in patients with melanoma and breast cancer (33, 45). Because the antibody response seems to be polyclonal, antibody inactivation by anti-idiotypic antibodies has not been a problem and specific antibody levels have been maintained against GM2 by immunizations at 3- or 4-month intervals for over 2 years (45). However, even the most potent conjugate vaccines have not been able to induce consistent antibody responses against all antigens, and the titers are never as high as can be achieved with mAb administration. The results obtained here, demonstrating the ability of either approach to protect against tumor challenge and to eliminate micrometastases, in the absence of any detectable toxicity, argue strongly in favor of the careful use of either approach or the combination.

GM2-KLH and GD2-KLH have both proven consistently immunogenic and safe in melanoma patients, whereas GD3 (the major melanoma ganglioside)-KLH has not proven so immunogenic (reviewed in Ref. 47). Adjuvant therapy of melanoma might optimally include a bivalent conjugate vaccine (GM2-KLH plus GD2-KLH), a humanized anti-GD3 mAb, or a combination of bivalent vaccine plus mAb.

Vaccines against infectious diseases do not prevent infection; they limit its spread from its point of contact. Postcontact boosts in antibody titers, even in protected hosts, attest to active infection at the contact site. This is most striking when time has elapsed because the original infection and antibody titers have fallen to low levels but rise to protective levels within 4–7 days, preventing symptomatic infection. In patients with cancer, we see the adjuvant setting (after removal of the primary cancer or positive lymph nodes) as being quite similar to the picture in patients being reexposed to infectious diseases. The primary targets in both cases are circulating pathogens and microscopic spread, and in the case of infectious diseases, antibodies are the primary method of protection. We demonstrate here, with passively administered mAbs and vaccine-induced antibodies against the defined cancer antigen GD2 ganglioside, that antibodies can also protect mice against circulating syngeneic tumor cells and micrometastases. If antibodies of sufficient titer and potency to eliminate circulating cancer cells and micrometastases could be maintained in cancer patients as well, even metastatic cancer would have quite a different implication. With continuing showers of metastases no longer possible, aggressive treatment of primary and metastatic sites might result in long-term control.

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